Toxicology Letters 219 (2013) 203–210

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Aberrant activation of M phase proteins by cell proliferation-evoking carcinogens after 28-day administration in rats

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HIGHLIGHTS

- This study aimed to identify early prediction markers of carcinogens in rats.
- Cellular distribution of cell cycle proteins was analyzed after 28-day treatment.
- Cell proliferation-evoking carcinogens induced activation of M phase proteins.
- Carcinogens lacking proliferative activity did not have these effects.
- Cell proliferation and M phase proteins might functions as an early prediction unit.

ARTICLE INFO

Article history:
Received 19 January 2013
Received in revised form 12 March 2013
Accepted 15 March 2013
Available online 25 March 2013

Keywords:
Carcinogen
M phase
Cell proliferation
Immunohistochemical analysis

ABSTRACT

We have previously reported that hepatocarcinogens increase liver cells expressing $p21^{Cip1}$, a G_1 checkpoint protein and M phase proteins after 28-day treatment in rats. This study aimed to identify early prediction markers of carcinogens available in many target organs after 28-day treatment in rats. Immunohistochemical analysis was performed on Ki-67, $p21^{Cip1}$ and M phase proteins [nuclear Cdc2, phospho-Histone H3 (p-Histone H3), Aurora B and heterochromatin protein 1α (HP1α)] with carcinogens targeting different organs. Carcinogens targeting thyroid (sulfadimethoxine; SDM), urinary bladder (phenylethyl isothiocyanate), forestomach (butylated hydroxyanisole; BHA), glandular stomach (catechol; CC), and colon (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and chenodeoxycholic acid) were examined using a non-carcinogenic toxicant (caprolactam) and carcinogens targeting other organs as negative controls. All carcinogens increased Ki-67$^+$, nuclear Cdc2$^+$, p-Histone H3$^+$ or Aurora B$^+$ carcinogenic target cells, except for both colon carcinogens, which did not increase cell proliferation. On the other hand, $p21^{Cip1+}$ cells increased with SDM and CC. HP1α responded only to BHA. Results revealed carcinogens evoking cell proliferation concurrently induced cell cycle arrest at M phase or showing chromosomal instability reflecting aberration in cell cycle regulation, irrespective of target organs, after 28-day treatment. Therefore, M phase proteins may be early prediction markers of carcinogens evoking cell proliferation in many target organs.

1. Introduction

In general, the method for evaluating carcinogenicity is a bioassay in which rodents are treated with a chemical for their entire 1.5- or 2-year lifespan. Carcinogenicity studies using experimental animals are time-consuming, expensive, and use many animals. However, there is no commonly rapid means for evaluating the carcinogenic potential of chemicals. Alternative animal models using medium-term carcinogenesis models (Tamano, 2010) or genetically modified animals using transgenic or gene-targeting technologies (Eastin, 1998) are also expensive and time-consuming or have limited target organs. Toxicogenomic approaches for the prediction of carcinogenic potential in each target organ appear promising. However, they are also expensive and require some integrative methodologies between different laboratories sharing an expression database (Uehara et al., 2011).

Development of nuclear enlargement is sometimes observed in carcinogenic target cells after repeated administration of

Abbreviations: SDM, sulfadimethoxine; PEITC, phenylethyl isothiocyanate; BHA, butylated hydroxyanisole; CC, catechol; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; CDCA, chenodeoxycholic acid; p-Histone H3, phospho-Histone H3; HP1α, heterochromatin protein 1α; CL, caprolactam.

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http://dx.doi.org/10.1016/j.toxlet.2013.03.012

carcinogens, irrespective of genotoxic potential, from the early stages of exposure in experimental animals (Adler et al., 2009; Allen et al., 2004). This nuclear enlargement is typically observed in the liver and kidney. It is often termed cytomegaly in cases of liver cells characterized by the presence of hepatocytes that are enlarged because of increased cytoplasmic volume, and karyomegaly when it occurs in renal tubular cells. Recent studies have shown that ochratoxin A, a representative renal carcinogen that can typically induce karyomegaly, induces aberrant expression of cell cycle-related proteins in the proximal tubular areas of the outer stripe of the outer medulla with karyomegaly (Adler et al., 2009). Generation of karyomegaly/cytomegaly suggests cell cycle aberration causing chromosomal instability through nuclear division during mitosis. Aberrant mitosis, such as chromosomal missegregation and cytokinesis failure occurring as a result of checkpoint dysfunction of the cell cycle, can induce tetraploidy/aneuploidy (Ichijima et al., 2010). This suggests that this aberrant expression of cell cycle-related proteins may eventually cause carcinogenicity in association with the development of chromosomal instability. Therefore, we hypothesize that an early event, which disrupts cell cycle regulation, triggers the carcinogenic response in the molecular mechanism responsible for the development of cytomegaly/karyomegaly.

We have previously analyzed cell cycle-related proteins in a 28-day study of repeated hepatocarcinogen administration to induce cytomegaly in rats (Yafune et al., 2013). These responses suggested hepatocarcinogens, irrespective of cytomegaly-inducing potential, induced an increase in the liver cell population immunoreactive for $p21^{Cip1}$ and Aurora B, suggestive of those undergoing G_1 arrest and chromosomal instability, respectively. We also found that hepatocarcinogens that evoke cell proliferation might cause M phase arrest of liver cells, judging from increased cell population expressing nuclear Cdc2, phospho-Histone H3 (p-Histone H3), and heterochromatin protein 1α (HP1α), accompanied with apoptosis. The obtained results suggested that a combination of these cell cycle proteins might be an early prediction battery of markers of hepatocarcinogens in a 28-day treatment scheme in rats.

There is a need for an available prediction tool to assess the carcinogenic potential of chemicals. To establish a short-term carcinogenicity screening system, it is reasonable to focus on common cellular responses in specific target organs. In the present study, based on our previous results on hepatocarcinogens, expression of these candidate proteins was explored in other target organs, including the thyroid, urinary bladder, forestomach, glandular stomach and colon, after 28-day treatment with organ-specific carcinogens in rats.

2. Materials and methods

2.1. Chemicals

Butylated hydroxyanisole (BHA; CAS No. 25013-16-5, ≥98.0%), caprolactam (CL; CAS No. 105-60-2, 98%), and catechol (CC; CAS No. 120-80-9, >99.0%) were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Chenodeoxycholic acid (CDCA; CAS No. 474-25-9, ≥98.0%) and phenylethyl isothiocyanate (PEITC; CAS No. 2257-09-2, ≥97.0%) were obtained from Tokyo Chemical Industry Corporation (Tokyo, Japan). Sulfadimethoxine sodium salt (SDM; CAS No. 122-11-2) was obtained from Sigma–Aldrich Corporation (St. Louis, MO, USA). 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP; CAS No. 105650-23-5, ≥98.0%) was obtained from Nard Institute (Hyogo, Japan).

2.2. Animal experiments

Five-week-old male F344/NSlc rats were purchased from Japan SLC, Inc. (Shizuoka, Japan) and acclimatized to a powdered basal diet (CRF-1 diet; Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum*. They were housed in stainless steel cages in a barrier-maintained animal room on a 12 h light–dark cycle and conditioned at 23 ± 3 °C with relative humidity of 50 ± 20%. After a 1-week acclimatization period, animals were randomized into groups of 10 each and treated with carcinogens or non-carcinogens for 28 days.

Animals were treated with carcinogenic doses of carcinogens targeting either the thyroid, urinary bladder, forestomach, glandular stomach or colon for 28 days. Groups received either SDM (1000 ppm in drinking water) targeting the thyroid, PEITC (1000 ppm in diet) targeting the urinary bladder, BHA (20,000 ppm in diet) targeting the forestomach, CC (8000 ppm in diet) targeting the glandular stomach, or CDCA (1000 ppm in diet) or PhIP (400 ppm in diet) targeting the colon. The dose of SDM and CDCA has been shown to promote carcinogenesis in the thyroid and colon, respectively, in rats (Ghia et al., 1996; Imai et al., 2004). With regard to PEITC, BHA, CC and PhIP, the dose has been shown to induce tumors in each target organ (Hagiwara et al., 2001; Ito et al., 1991; Kaneko et al., 2002; Sugiura et al., 2003). CL (10,000 ppm in diet) was selected as a non-carcinogenic control compound, exhibiting positivity in some genotoxicity studies (IARC, 1999). This compound has shown no carcinogenic effect in any organs with ≥7500 ppm in diet (Fukushima et al., 1991; NTP, 1982). Untreated control animals were given basal diet and tap water *ad libitum* for 28 days.

One day after the 28-day treatment, all animals were sacrificed by exsanguination from the abdominal aorta under deep anesthesia using CO_2/O_2, and target organs were removed. Target organs were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer solution (pH 7.4; Wako Pure Chemicals Industries, Ltd.). At necropsy, the urinary bladder was inflated by transurethral instillation of a paraformaldehyde solution, and the stomach and colon were instilled with a paraformaldehyde solution to facilitate mucosal fixation. The following samples were taken from fixed tissues and prepared for paraffin embedding: bilateral lobes of the thyroid; two longitudinal slices of the urinary bladder; three longitudinal slices of the stomach including forestomach and glandular stomach; and three cross cut pieces each from proximal, medial, and distal portions of the colon.

All procedures in this study were conducted in compliance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, June 1, 2006) and according to the protocol approved by the Animal Care and Use Committee of the Tokyo University of Agriculture and Technology.

2.3. Histopathology and immunohistochemistry

Three micrometer sections of paraffin-embedded tissues from the thyroid, urinary bladder, stomach, and colon were stained with hematoxylin and eosin for histopathological examination and subjected to immunohistochemistry.

Immunohistochemistry was performed using the Vectastain® Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA) with 3,3′-diaminobenzidine/H_2O_2 as the chromogen. The following primary antibodies were used: Ki-67 (mouse monoclonal antibody, 1:50; Dako, Glostrup, Denmark), $p21^{Cip1}$ (mouse monoclonal antibody, 1:100; Abcam, Cambridge, UK), Cdc2 (mouse monoclonal antibody, 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Aurora B (rabbit polyclonal antibody, 1:200; Abcam), p-Histone H3 (Ser 10 phosphorylated; rabbit polyclonal antibody, 1:50; Santa Cruz Biotechnology, Inc.), and HP1α (rabbit polyclonal antibody, 1:200; Cell Signaling Technology, Inc., Danvers, MA, USA). These antigens were selected based on our previous results (Yafune et al., 2013). Antigen retrieval was performed in an autoclave for 10 min at 121 °C in 10 mM citrate buffer (pH 6.0) for Ki-67 and p-Histone H3 and in a microwave for 10 min at 90 °C in 10 mM citrate buffer (pH 6.0) for $p21^{Cip1}$ and HP1α. Sections were counterstained with hematoxylin for microscopic examination.

2.4. Analysis of immunoreactivity

In the thyroid and urinary bladder, immunostained cells in the follicular area (thyroid) and mucosal area (urinary bladder) were counted in eight randomly selected areas per animal (four areas per tissue section) at magnifications of 400× in the former and 200× in the latter. In the forestomach, because cells immunoreactive for Ki-67, Aurora B, or HP1α were diffusely distributed in the basal cell layer, vertical length of positive cell distribution from the basement membrane was measured in 10 randomly selected areas per animal at 200× magnification. $p21^{Cip1+}$, nuclear $Cdc2^+$ or p-Histone $H3^+$ cells in the forestomach were counted in 10 randomly selected areas in the mucosa per animal at 200× magnification. In the glandular stomach, immunoreactive cells were counted in 10 randomly selected glands per animal at 200× magnification. In the colon, immunoreactive cells were counted in 10 randomly selected crypts per animal that were located close to the lamina muscularis mucosa and demonstrated a cross sectional view at 200× magnification.

Total cells were measured by counting all nuclei in each selected field in the thyroid and urinary bladder mucosa using WinROOF image analysis and measurement software (version 6.4.2., Mitani Corporation, Fukui, Japan). The percentage of immunoreactive cells was determined in each field. In the forestomach, mean vertical length of the distribution of Ki-67^+, Aurora B^+ or HP1α^+ cells within the mucosa was estimated from 10 fields and expressed as vertical length (μm). $p21^{Cip1+}$, nuclear $Cdc2^+$ or p-Histone $H3^+$ cell counts were expressed as numbers per 1000 μm of epithelial layer length in each field. In the glandular stomach, mean number of immunoreactive cells of 10 glands/animal was estimated and expressed as cells per gland. In the colon, percentage of immunoreactive cells was determined in each crypt by selecting 10 glands using WinROOF image analysis and measurement software.